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(54) Title: PROTEINS DERIVED FROM WHITE SPOT SYNDROME VIRUS AND USES THEREOF

(57) Abstract: The present invention relates to the isolation and characterization of four major proteins derived from White Spot Syndrom Virus (WSSV) having an estimated size of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24) and 19 kDa (VP19) and their use in the manufacture of a vaccine to protect crustaceans against infections with WSSV. The invention further provides for nucleotide sequences encoding said proteins and their use in recombinant production of said proteins. In addition the invention provides for antibodies raised against said proteins, their use in passive vaccination and diagnostic kits comprising said nucleic acids or said antibodies.

PROTEINS DERIVED FROM WHITE SPOT SYNDROME VIRUS AND USES THEREOF

The present invention relates to proteins derived from White Spot Syndrome virus, nucleic acid sequences encoding them and use of said proteins in the manufacture of a vaccine for prophylaxis and/or treatment of White Spot Syndrome in crustaceans.

White Spot Syndrome Virus (WSSV) is a major viral disease in shrimp in large areas of Southeast Asia. The virus has a wide host range among crustaceans (Flegel, 1997) and there is little genetic variation among isolates (Lo et al, 1999). Electron microscopy (EM) studies showed that the virions are enveloped and have a rod to bullet shaped appearance of about 275 nm in length and 120 nm wide with a tail-like appendage at one end. Nucleocapsids, which have lost their envelope, have a crosshatched appearance and a size of about 300 nm x 70 nm (Wongteerasupaya et al., 1995). This virion morphology, its nuclear localisation and its morphogenesis are reminiscent of baculoviruses in insects (Durand et al., 1997). Originally, WSSV has been classified as an unassigned member of the Baculoviridae family (Francki et al., 1991)hence the virus has been referred to as Systemic Ectodermal Mesodermal Baculo virus (SEMBV) or White Spot Baculo virus (WSBV). At present WSSV is no longer accepted into this family (Murphy et al., 1995) due to lack of molecular information. The double stranded viral DNA has a size of well over 200kb as derived from restriction endonuclease analysis (Yang et al., 1997).

An outbreak of WSSV in cultured shrimp in Southeast Asia causes mass mortality among the shrimp. The disease is characterised by white spots on the carapace, appendages and cuticle and reddish coloration of the hepatopancreas of the shrimp. The infected shrimps show signs of lethargy and a rapid reduction in food consumption and within 3 to 5 days these shrimps die. An outbreak of WSSV leads to heavy losses in the industry of cultured shrimp and as a consequence there is a strong need for vaccines that can protect against WSSV infections. The identification and characterisation of major structural WSSV proteins that can be used in such a vaccine would provide the means to develop such vaccines.

Four major proteins of WSSV have been identified which have been designated VP28 (28 kDa), VP26 (26 kDa), VP24 (24 kDa) and VP19 (19 kDa) due to their molecular weight estimated from their mobility in Coomassie Brilliant Blue-stained SDS-PAGE gels. VP26 and VP24 are nucleocapsid proteins, whereas VP28 and Vp19 are envelope proteins. The N-terminal amino acid residues of the WSSV

proteins were obtained by protein sequencing, and were used to identify their genes (*vp28*, *vp26*, *vp24*, *vp19*, respectively) on the WSSV genome. The open reading frame (ORF) of *vp26* comprises 555 nucleotides and is depicted in Fig.2b (SEQ ID NO:1) together with the deduced amino acid sequence of VP26, which is depicted as an 184 amino acid residues sequence (SEQ ID NO:3) in Fig.2b. A second open reading frame of *vp26* comprises 612 nucleotides and is depicted in SEQ ID NO:9 together with the deduced amino acid sequence consisting of 204 residues, which is separately depicted as SEQ ID NO:10. The open reading frame of *vp28* comprises 615 nucleotides (SEQ ID NO:2) and is depicted in Fig.2c together with the deduced amino acid sequence (SEQ ID NO:4). The deduced amino acid sequence of VP28 is 204 amino acids. Both VP26 and VP28 contain a putative transmembrane domain at the N-terminus and many putative N- and O- glycosylation sites. The ORF of the genes *vp26* and *vp28* coded for proteins with a theoretical size of 20 kDa and 22 kDa respectively. The theoretical amino acid sequence of VP26 and VP28 was confirmed by direct protein sequencing. The theoretical sizes of VP26 and VP28 differ 6 kDa from the size estimated by their mobility in Coomassie Brilliant Blue-stained SDS-PAGE gels. This size difference could be explained by posttranslational modifications such as glycosylation, phosphorylation, etc. The N-terminal amino acid sequence of VP24 and the partial amino acid sequence of VP19 are depicted in SEQ ID NO: 5 and 6 respectively. The complete open reading frame of *vp24* comprises 627 nucleotides and is depicted in SEQ ID NO:11 together with the deduced amino acid sequence of VP24. The deduced amino acid sequence of VP24 has 208 residues and is separately depicted in SEQ ID NO:12. The four proteins and their respective nucleotide sequences are specific for WSSV.

The present invention provides for the first time the means to produce recombinant vaccines to protect crustaceans against infection with WSSV. The four major proteins VP28, VP26, VP24 and VP19 of WSSV which have been identified and characterised were found to be suitable for use in the manufacture of a subunit vaccine to protect crustaceans against infections with WSSV. The cloning and characterisation of the nucleotide sequences of the present invention provides for the production of these structural proteins of the WSSV using recombinant technology techniques. In this way, recombinant structural WSSV proteins can be obtained, which are substantially free from other WSSV proteins. The isolated structural WSSV proteins can be used to manufacture subunit vaccines to protect crustaceans against infection of WSSV. Alternatively the nucleotide sequences encoding the structural proteins of the WSSV can be used to manufacture vector vaccines to protect crustaceans against the infection with WSSV. The nucleotide sequences of the present invention can furthermore be used for diagnostic purposes, for instance to detect the presence of WSSV in the field. Additionally, the WSSV

proteins of the present invention can be used to produce WSSV specific antibodies. These antibodies can be used to produce WSSV vaccines for passive immunisation of the crustaceans. The antibodies can also be used for diagnostic purposes such as the detection of WSSV in crustaceans or in the field.

Thus in a first object the invention provides for the structural proteins of WSSV. More specifically the invention provides for structural proteins VP24, VP26, VP28 and VP19. In particular the invention provides for protein VP26 having an amino acid sequence depicted in Fig. 2b (SEQ ID NO:3) or a derivative sequence thereof, such as for example SEQ ID NO:10, and VP28 having an amino acid sequence depicted in Fig. 2c (SEQ ID NO:4) or a derivative sequence thereof. The invention further provides for protein VP24 comprising the N-terminal amino acid sequence M H M W G V Y A A I L A G L T L I L V V I S I V V T N I E L N K K L D K K D K depicted in SEQ ID NO:5 or a derivative thereof, and protein VP19 comprising the partial amino acid sequence I V L I S I (G/V) I L V L A V M N V (P/A/T) M G P K K D S depicted in SEQ ID NO:6 or a derivative thereof. Preferably a protein VP24 has the amino acid sequence as depicted in SEQ ID NO:12 or a derivative sequence thereof. It must be understood that proteins having a derivative sequence of the amino acid sequences depicted in SEQ ID NO 3, 4, 5, 6, 10 or 12 are also within the scope of the present invention. For the purpose of this invention a derivative of the protein amino acid sequence is understood to be an amino acid sequence that comprises alterations compared to the amino acid sequence depicted in SEQ ID NO:3, 4, 10 or 12 or the partial sequences depicted in SEQ ID NO:5 or 6, whereby said alterations do not affect the antigenic or immunogenic characteristics of the proteins. For the purpose of this invention antigenic characteristics of the proteins are understood to be the ability of the proteins to raise antibodies that are capable of recognising and/or reacting with said WSSV proteins. Immunogenic characteristics are understood to be the ability of the proteins to induce a protective response in the crustaceans against WSSV infections.

The alterations that can occur in a sequence according to the present invention could for instance result from conservative amino acid substitutions, deletions, insertions, inversions or additions of (an) amino acid(s) in the overall sequence. Amino acid substitutions that are expected not to alter the immunological properties have been described. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science, 1985, vol. 227, 1435-1441) and determining the functional similarity between proteins and peptides having sequence homology. Several computerprograms such as FASTA, TFASTA, BLAST and the like are available to determine sequence

homology between a protein or peptide with given amino acid sequence and a derivative thereof; the optical match area between the sequences can be automatically determined by these programs. Thus derivative proteins according to the invention are still capable to raise antibodies that recognise and can react with the structural WSSV proteins, or to induce a protective response in vaccinated crustaceans that protects them against WSSV infection. Other derivative proteins that can be used according to the invention are fragments of the WSSV proteins, provided said fragments are still capable to raise antibodies that recognise and can react with the structural WSSV proteins, or to induce a protective response in vaccinated crustaceans to protect them against WSSV infection.

In a second aspect the invention provides for a nucleic acid sequence encoding one or more structural proteins of WSSV. More preferably the present invention provides for a nucleic acid sequence encoding the major structural proteins VP24, VP26, VP28 and/or VP19, respectively. In particular the present invention provides for a nucleic acid sequence of *vp26, vp28* and *vp24* depicted in SEQ ID NO:1 or 9, SEQ ID NO:2 or SEQ ID NO:11 encoding VP26, VP28 and VP24 respectively. The respective nucleotide sequences start with the ATG codon encoding the first M residue of the deduced amino acid sequence up to the codon encoding the C-terminal amino acid residue. It must be understood that for the purpose of this invention nucleic acid sequences that have sequence homology with the sequences depicted in SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO 9 or SEQ ID NO:11 are also within the scope of the invention. The sequence homology for the purpose of this invention is considered to be at least 70%, preferably 75%, more preferably 80%, even more preferably 85%. Highly preferred are nucleic acid sequences that have sequence homology with the sequences depicted in SEQ ID NO:1, 2, 9 or 11 of at least 90% more preferably 95%.

For the purpose of this invention sequence homology is determined by comparing the nucleotide sequence of interest with the corresponding part of the sequence depicted in SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:11. For the purpose of this invention the percentage sequence homology is defined as the percentage of identical nucleotides between the compared sequences. The sequence homology can be determined for instance by computerprograms such as BLAST N and the like. These programs automatically determine the optimal match area.

Nucleic acid sequences having sequence homology according to the invention can easily be isolated with one of the sequences depicted in SEQ ID NO 1, 2, 11 or 9 or with fragments of this sequence from closely related WSSV strains using routine cloning and hybridisation techniques. For this purpose hybridisation is carried out under stringent, preferably highly stringent conditions. Stringent hybridisation conditions are understood to be washing conditions of 1 x SSC, 0.1%SDS at a temperature

of 65°C; highly stringent conditions refer to washing conditions in which the concentration SSC is being lowered towards 0.3 x SSC. The specific information should not be so narrowly interpreted so as to require exclusion of erroneously identified bases. The specific sequences disclosed herein can be readily used to isolate homologous nucleotide sequences from other strains.

A nucleic acid sequence that has sequence homology with one of the sequences depicted in SEQ ID No's 1, 2 or 11 encodes a protein having an amino acid sequence which comprises alterations compared to one of the amino acid sequences depicted in SEQ ID NO's 3, 4, 10 or 12 or one of the partial amino acid sequences depicted in SEQ ID No's 5 and 6, whereby said alterations do not affect the antigenic or immunogenic properties of said protein. An example of such homologous nucleotide sequence encoding a VP26 protein is the nucleotide sequence depicted in SEQ ID NO:9, which encodes for a VP26 protein having alterations compared to the amino acid sequence depicted in SEQ ID NO 3.

The WSSV proteins according to the invention can be obtained via standard biochemical isolation and purification methods or they can be prepared via general recombinant technology. The nucleotide sequences according to the invention are particularly suitable to be used for the recombinant production of structural WSSV proteins, substantially free from other WSSV proteins. The nucleotide sequences are incorporated into a suitable expression vector capable of expressing the proteins, transforming a suitable host cell with said expression vector and culturing the host cell in a suitable medium. The expressed proteins can be isolated and purified from the cells or the medium. Suitable expression vectors are, amongst others, plasmids, cosmids, viruses and YAC's (Yeast Artificial Chromosomes) which comprise the necessary control regions for replication and expression. The expression vector can be brought to expression on a host cell. Suitable host cells are, for instance, bacteria, yeast cells, insect cells and mammalian cells. Such expression techniques are well known in the art (Sambrooke et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989; King and Possee, 1992).

In a third aspect the invention provides for a vaccine comprising one or more of the structural virion proteins VP24, VP26, VP28 or VP19 of WSSV and a pharmaceutical acceptable carrier. More specifically, a vaccine according to the invention comprises virion protein VP24, VP26, VP28 or VP19 or a combination of two or more of said proteins. Preferably a vaccine according to the invention comprises VP24 comprising either the amino acid sequence depicted in SEQ ID NO12 or the N-terminal amino acid sequence depicted in SEQ ID NO:5 or a derivative sequence of either sequences, or VP26 comprising the amino acid sequence depicted in SEQ ID NO:3, SEQ ID NO:10, or a derivative sequence of either

sequence, or VP28 comprising the amino acid sequence depicted in SEQ ID NO:4 or a derivative sequence thereof, or VP19 comprising the N-terminal amino acid sequence depicted in SEQ ID NO:6 or a derivative sequence thereof, or a combination of two or more of said proteins. More preferably a vaccine according to the invention comprises WSSV proteins VP26 and VP28, and optionally VP24.

In addition, the nucleic acid sequences according to the invention can be used to manufacture a vector vaccine to vaccinate crustaceans against WSSV infections. A vector vaccine is understood to be a vaccine in which a live, attenuated bacterium or virus has been modified so that they contain one or more heterologous nucleotide sequences inserted into their genetic material. These so called vector bacteria or viruses are capable of co-expressing the heterologous proteins encoded by the inserted nucleotides. Thus in a fourth aspect the invention provides for a vector vaccine comprising a live attenuated bacteria or virus and a pharmaceutical acceptable carrier, in which said bacteria or virus has been modified to comprise in its genetic material one or more of the nucleotide sequences of the present invention.

A vaccine according to the invention can be used to protect crustaceans such as shrimps including but not limited to members from the *Penaeidae* family such as for example *P.monodon, P.vannamei, P.chinensis, P.merguensis,* or *Metapeaeus spp.*; prawns including but not limited to members from the Palaemonidae family such as for example *Macrobrachium spp.* or *Palaemon spp.*; lobsters including but not limited to members from the Palinuridae and Nephropidae family such as for example *Calinectes spp., Palinurus spp., Panuliris spp.* or *Homarus spp.*; crayfish including but not limited to members from the Astacidae family examples of which are *Astacus spp., Procambarus spp.*, and *Oronectes spp.*; and crab including but not limited to members from the Cancridae and Portuidae family, examples of which are *Cancer spp., Callinectes spp., Carcinus spp.* and *Portunus spp.*

A vaccine according to the invention can be prepared according to techniques well known to the skilled practitioner and described for instance in Remington's Pharmaceutical Sciences, 18th edition (1990), eds. A.R. Gennaro et al., chapter 72, pp. 1389-1404, Philadelphia College of Pharmacy and Science.

Vaccines according to the invention comprise an effective amount of one or more proteins, vector bacteria or virus according to the invention, and a pharmaceutical acceptable carrier. The term "effective " as used herein is defined as the amount sufficient to induce a protective response in the crustaceans. The amount of vector or protein will depend on the type of vector or protein, the route of

administration, the time of administration, the species to be vaccinated as well as age, general health, temperature and diet.

In general, a dosage of 0.01 to 1000 µg protein per animal, preferably 0.5 to 500 µg, more preferably 0.1 to 100 µg protein per animal can be used. In case of viral vector vaccines in general a dosage of 103 to 108 pfu (plaque forming units) per animal can be used.

Pharmaceutically acceptable carriers that are suitable for use in a vaccine according to the invention are sterile and physiologically compatible such as for example sterile water, saline, aqueous buffers such as alkali metal phosphates (e.g. PBS), alcohol's, polyols and the like. In addition a vaccine according to the invention may comprise other additives such as adjuvants, stabilisers, anti-oxidants, preservatives and others.

Suitable adjuvants include but are not limited to aluminium salts or gels, carbomers, non-ionic blockcopolymers, tocopherols, monophospheryllipid A, muramyldipeptide, oil emulsions, glucans, cytokines, saponins such as Quil A, and the like. The amount of adjuvant added depends on the nature of the adjuvant itself.

Suitable stabilisers for use in a vaccine according to the invention include but are not limited to carbohydrates such as sorbitol, mannitol, starch, sucrose, dextrin, and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Suitable preservatives include, amongst others thimerosal and merthiolate.

The vaccines according to the invention can be administered via injection, immersion, dipping, spray or aerosol, or per oral. Preferably the vaccine is administered to the crustaceans via immersion or per oral, especially in case of commercial aquaculture farms.

For oral administration the vaccine is preferably mixed with a suitable carrier for oral administration i.e. cellulose, food or a metabolizable substance such as alpha-cellulose or different oils of vegetable or animals origin. Particularly preferred food carriers for oral delivery of the vaccine according to the invention are live-feed organisms which are able to encapsulate the vaccine. Suitable live-feed organisms include but are not limited to plankton-like non-selective filter feeders preferably members of Rotifera, *Artemia*, and the like. Highly preferred is the brine shrimp *Artemia sp.*.

The proteins according to the invention can be used for the production of antibodies, using the general techniques available to the practitioner in the field. Preferably the proteins are used to produce specific monoclonal antibodies. Antibodies according to the invention can be prepared according to standard techniques. Procedures for immunising animals, e.g. mice, with proteins and selection of

hybridomas producing proteins specific monoclonal antibodies are well known in the art (see for example Cligan et al. (eds), Current protocols in Immunology 1992; Kohler and Milstein, Nature 256, pp. 495-497, 1975; Steenbakkers et al., Mol. Biol. Rep. 19, pp. 125-134, 1994). The obtained antibodies may be utilised in diagnostics to detect WSSV in the field or to detect the presence of WSSV in the crustaceans. The nucleotide sequences according to the invention are also suitable for use in diagnostics. Said sequences or fragments thereof can be used in for instance PCR technology to detect the presence of WSSV in the field, or in the crustaceans. Thus, in another aspect, the present invention provides for a diagnostic kit comprising one or more nucleotide sequences or antibodies according to the invention.

The antibodies raised against the proteins VP28, VP26, VP24 and VP19 according to the invention can further be used to manufacture antibody vaccines for the passive immunisation of the crustaceans. Thus, in a furher aspect, the present invention provides for a vaccine for passive immunisation against WSSV said vaccine comprising antibodies raised against either VP28, VP26, VP24, or VP19 or a combination of two or more of said proteins. Such a vaccine can be prepared using standard techniques, as mentioned above. Preferably a vaccine for oral administration of the antibodies is prepared, in which the antibodies are mixed with an edible carrier such as fish food. More preferably, the vaccine is prepared from antibodies prepared in chicken eggs (IgY antibodies).

The following examples are to illustrate the invention and should not be interpreted to limit the invention in any way.

LEGENDS

Figure 1

WSSV proteins. (A) TEM picture of negatively stained intact virions. (B) TEM picture of negatively stained WSSV nucleocapsids. (C) 15% coomassie stained SDS PAGE gel of purified WSSV. Lane 1: Low molecular weight protein marker. Lane 2: purified "WSSV particles" from uninfected shrimp. Lane 3: purified WSSV particles. Lane 4: purified WSSV nucleocapsids.

Figure 2

Nucleotide sequence of WSSV VP26 and VP28. (A) Location of VP26 and VP28 on WSSV genomic fragments. (B) Nucleotide and protein sequence of VP26 and (C) of VP28. The ORF of *vp26* and *vp28*,

respectively, start at the ATG codon encoding the first M residue of the deduced amino acid sequence. The N-terminal sequences amino acids are bold faced; the location of putative N-glycosylation sites is underlined and of putative O-glycosylation sites double underlined. The nucleotide sequence of degenerated primer positions on VP28 are in italics.

Figure 3

Hydrophobicity plots of (A) VP26 and (B) VP28.

Figure 4

Baculovirus expression of WSSV structural proteins in insect cells analyzed in a 15% SDS PAGE gel and western blot. (A) Coomassie stained gel with extracts of *Sf*21 cells. Lane 1: Low molecular weight protein marker. Lane 2: mock infection. Lane 3: *Ac*MNPV-wt infection. Lane 4: *Ac*MNPV-GFP infection. Lane 5: *Ac*MNPV-WSSVvp26 infection. Lane 6: *Ac*MNPV-WSSVvp28 infection. Lane 7: WSSV. (B) Western blot using a polyclonal antibody against purified WSSV.

Figure 5

Neutralisation of WSSV in shrimp by antiserum raised against structural protein VP28. Negative control: shrimps receiving NaCl solution. Positive control: shrimps receiving WSSV but no antiserum. Pre-immune serum: shrimps receiving WSSV and pre-immune serum. VP28 antiserum: shrimps receiving virus and anti-VP28 antiserum.

Figure 6

Vaccination of shrimps with WSSV proteins. Negative control: shrimps receiving NaCl solution. Positive control: shrimps receiving NaCl and WSSV. Group 3: shrimps vaccinated with VP24. Group 4: shrimps vaccinated with VP26c. Group 5: shrimps vaccinated with VP28. Group 6: shrimps vaccinated with a mixture of VP24, VP26c and VP28.

EXAMPLES

Methods

White Spot Syndrome Virus production and purification

The virus used in this study was isolated from infected *Penaeus monodon* shrimp from Thailand. Infected tissue was homogenized in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4). After centrifugation at 1,700 x g for 10 min the supernatant was filtered (0.45 µm filter). The filtrate was injected intramuscularly into healthy *P. monodon* in the lateral area of the fourth abdominal segment to initiate infection. After 4 days haemolymph was withdrawn from moribund shrimp and mixed with modified Alsever solution (Rodriguez *et al.*, 1995) as an anticoagulant. After dilution in TNE (20 mM Tris-HCl, 400 mM NaCl, 5 mM EDTA, pH 7.4), the haemolymph was clarified from haemocytes at 1,700 x g for 10 min at 4°C. The virus particles were sedimented by centrifugation at 45,000 x g at 4°C for 1 h and suspended from the pellet in TN.

The virus envelope was removed from the virus particles by treatment with Nonidet P40 (NP40). One percent NP40 was added to virus solution and incubated for 30 min at room temperature with gentle rocking. The nucleocapsids were sedimented at 80,000 x g for 30 min at 4°C. The pellet was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

SDS-PAGE of virion suspensions

For protein analysis, the WSSV virion preparations (enveloped virions, the nucleocapsids and the negative control) were analyzed in a 15% SDS-PAGE gel. Proteins were visualised in SDS-PAGE gel using coomassie brilliant blue staining.

Electron microscopy

For transmission electron microscopy (TEM), virus suspension were mounted on formvar-coated, carbon-stabilised nickel grids (400 mesh), negatively stained with phosphotungstic acid (2% PTA). The specimens were examined by use of a Philips CM12 electron microscope.

Nucleic acid purification

Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/ml) and sarcosyl (1%) at 45°C for 3 h, followed by phenol/chloroform extraction and dialysis against TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The purity and concentration of the DNA was determined by agarose gel electrophoresis using a marker.

Plasmid constructions

WSSV subgenomic fragments were cloned into pBluescript SK+ (Stratagene) and transformed into *E. coli* DH5α using standard techniques (Sambrook *et al.,* 1989). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis and colony lifting were carried out according to standard protocols (Sambrook *et al.,* 1989). PCR was performed using custom designed and synthesised primers. DNA encoding the N-terminal of *vp28* was amplified by PCR from total WSSV DNA using degenerated primers based on the N-terminal amino acid sequence of VP28. The forward primer used was 5' CA*GAATTC*TCDATNGTYTTNGTNAC 3' (SEQ ID NO:7) and the reverse primer was 5' CA*GAATTC*ATGGAYYTNWSNTTYAC 3' (SEQ ID NO:8) with *Eco*RI sites (italics) (D = A, T or G; N = A, C, G, or T; Y = C or T; W = A or T; S = C or G). The N-terminal of *vp*24 was amplified by PCR from total WSSV DNA using a set of degenerate PCR primers based on the N-terminal amino acid sequence of VP24. 5' CA*GAATTC*ATGCAYATGTGGGGNGT 3' (SEQ ID NO:13) was used as forward primer, and 5' CA*GAATTC*YTTRTCYTTYTTRTCIARYTT 3' (SEQ ID NO:14) as reverse primer, both containing *Eco*RI sites (*italics*).

DNA sequencing and computer analysis

Plasmid DNA for sequencing was purified via the QIAprep Miniprep System or JETstar Plasmid Purification System (Qiagen, Inc.). Sequencing was performed using the universal pBluescript forward and reverse nucleotide primers and custom synthesised primers from both strands. Automatic sequencing was carried out using an Applied Biosystems automated DNA sequencer (Eurogentec, Belgium).

The generated sequences were analyzed with UWGCG computer programs (release 10.0). The DNA and the deduced amino acids sequences were compared with the updated GenBank/EMBL, SWISSPORT and PIR databases using the programs FASTA, TFASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et al.*, 1997).

Cells and viruses

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in Grace's insect medium (GIBCO BRL) supplemented with 10% foetal calf serum (FCS). The E2-strain of *Autographa californica* nuclear polyhedrosis virus (*Ac*MNPV) (Smith and Summers, 1982) was used as wild type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987; King and Possee, 1992).

Engineering of recombinants

The Bac-to-Bac system (GIBCO BRL) was employed to overexpress WSSV VP24 (SEQ ID NO 12) VP26 (SEQ ID NO 3), VP26c (SEQ ID NO 10) and VP28 (SEQ ID NO 4) in insect cells. To facilitate detection and titration of Bac-to-Bac recombinants upon infection of insect cells the Green Fluorescent Protein (GFP) gene was introduced into the pFastBac-DUAL vector downstream of the p10 promoter. The GFP gene was removed from plasmid pVL92GFP (Reilander *et al.*, 1996) after digestion of this plasmid with *Xba*I and *Kpn*I. The 700 bp GFP-containing fragment was isolated by agarose gel electrophoresis and GlassMAX purification (GIBCO BRL), blunt-ended using DNA polymerase and inserted into the *Sma*I site of multiple cloning region II of pFastBac-DUAL downstream of the p10 promoter. The resulting plasmid was named pFastBac-D/GFP and contained region I for insertion of a foreign gene downstream of the polyhedrin promoter. Recombinant virus expressing only the GFP from the p10 promoter was constructed according to the Bac-to-Bac system protocol (GIBCO BRL) and the virus was designated *Ac*MNPV-GFP.

PCR was performed on the WSSV plasmids containing the putative complete open reading frames (ORFs) of *vp26* (SEQ ID NO 1) and *vp28* (SEQ ID NO 2) introducing a *Bam*HI site at the 3' end of the ORFs and a *Hin*dIII site at the 5' end. *Vp26* (SEQ ID NO 1) and *vp28* (SEQ ID NO 2) were first cloned into the pET28a vector (Novagen), excised with *Bam*HI and *Not*I, and inserted downstream of the polyhedrin promoter of plasmid pFastBac-D/GFP. The resulting plasmids were named pFastBac-D/G-vp26 and pFastBac-D/G-vp28, respectively. *Vp*26c (SEQ ID NO 9) and *vp*24 (SEQ ID NO 11) were amplified by PCR on the plasmids containing the putative ORFs using primers introducing a *Bam*HI site at the 5' end and and *Eco*RI site on the 3' end. After digestion the ORFs of *vp*26c (SEQ ID NO 9) and *vp*24 (SEQ ID NO 11) were inserted downstream of the polyhedrin promoter of pFastBac-D/GFP, resulting in plasmids pFastBac-D/G-vp26c and pFastBac-D/G-vp24. Recombinant viruses expressing the GFP off the p10 promoter and VP24 (SEQ ID NO 12), VP26 (SEQ ID NO 3), VP26c (SEQ ID NO 10)

or VP28 (SEQ ID NO 4) off the polyhedrin promoter were constructed according to the Bac-to-Bac system protocol (GIBCO BRL) and the viruses were designated *Ac*MNPV-WSSVvp24, *Ac*MNPV-WSSVvp26, *Ac*MNPV-WSSVvp26c and *Ac*MNPV-WSSVvp28, respectively.

SDS-PAGE, protein sequencing and immunoblotting

Insect cells infected with wild type *Ac*MNPV and recombinant *Ac*MNPV expressing heterologous proteins (GFP, VP26, VP28) were analyzed in 15% SDS-PAGE gels. Proteins were visualized using coomassie brilliant blue staining. Semi dry blotting was performed onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a CAPS buffer (10 mM CAPS in 10% Methanol) or onto an Immobilon™-P (Millipore) using a Tris-Glycine buffer (25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3). Proteins were visualized on the PVDF membrane using coomassie brilliant blue staining. Major protein bands from WSSV virion preparations were excised from the filter and N-terminally sequenced (ProSeq. Inc., Massachusetts).

Immobulon-P membranes were blocked in 2% low-fat milk powder (Campina, the Netherlands) in TBS (0.2 M NaCl, 50 mM Tris-HCl, pH 7.4). Immunodetection was performed by incubated the blot in a polyclonal rabbit anti-WSSV serum (a gift from Prof. P.C. Loh, University of Honolulu, Hawaii) diluted 1:2000 in TBS with 0.2% low-fat milk powder for 1 h at room temperature. Subsequently, anti-rabbit antibody conjugated with horseradish peroxidase (Amersham) was used at a concentration of 1 : 2000 and detection was performed with an "Enhanced chemiluminescent-light detection kit" (Amersham).

VP28 polyclonal antibody

The major WSSV structural envelope protein VP28 was expressed in insect cells using baculovirus *Ac*MNPV-WSSVvp28 and purified using a Prepcell (Biorad) and a fraction collector. Fractions containing VP28 were collected and concentrated. The purified VP28 protein was injected in a rabbit to produce a polyclonal antibody. The antibody was tested on western blots containing purified WSSV virions and reacted well with VP28 from the WSSV virions. This VP28 antiserum was used in a WSSV neutralization experiment.

WSSV virus stock

A White Spot Syndrome Virus (WSSV) virus stock was generated by purifying virus from haemolymph of crayfish *Procambarus clarkii*, which were injected intramuscular with a low concentration WSSV one

week earlier. The haemolymph was purified over a continuous sucrose gradient and a virus band was removed. After pelleting of the virus, the virus was dissolved in TE (pH 7.5). The virus stock was stored at -70°C until use in the experiments.

Protein vaccination

The major WSSV structural envelope protein VP28 (SEQ ID NO 4) and nucleocapsid proteins VP26c (SEQ ID NO 10) and VP24 (SEQ ID NO 12) were expressed in insect cells using baculovirus *Ac*MNPV-WSSVvp28, *Ac*MNPV-WSSVvp26c and *Ac*MNPV-WSSVvp24, which express GFP off the p10 promoter and the WSSV structural proteins from the polyhedrin promoter. 3 days post infection the infected insect cells were harvested and disrupted by sonification. The supernatant was used to vaccinate *P. monodon.*

6 groups of shrimp were used in this experiment:

Group #	Group name	Vaccination	Booster	Challenge	# shrimp
1	Negative control	330 mM NaCl	330 mM NaCl	330 mM NaCl	10
2	Positive control	330 mM NaCl	330 mM NaCl	WSSV	10
3	VP28	VP28	VP28	WSSV	15
4	VP26c	VP26c	VP26c	WSSV	15
5	VP24	VP24	VP24	WSSV	15
6	MIX	mix	mix	WSSV	15

In the MIX equal volumes of the VP28, VP26c and VP24 solutions were mixed before injection. 5 days after vaccination, the shrimp obtain a booster injection. Two days later the challenge is performed by injection of WSSV (stock virus, see neutralisation experiment). After injection the shrimp were monitored for 6 days and dead shrimp were examined for the presence of WSSV by electron microscopy.

Results

Isolation of WSSV proteins for sequencing

Penaeus monodon shrimp were infected with WSSV by injection of a purified virus preparation intramuscularly. Four days after infection, virus was isolated from the haemolymph of the infected animals. As a negative control, haemolymph was taken from uninfected shrimps. These preparations

were analyzed by electron microscopy for the presence and purity of WSSV virions. In the samples of uninfected animals, no virus particles were observed, but in samples of the infected animals, many mainly enveloped virions were observed (Fig.1a). The viral envelope was removed from the virus particles after treatment with NP40 resulting in purified nucleocapsids (Fig. 1b), with a superficial segmented appearance characteristic for WSSV nucleocapsids (Durand *et al.*, 1997). The proteins of the enveloped virions and the nucleocapsids were separated by SDS-PAGE (Fig. 1c). Four major peptides were identified in virions with an apparent molecular mass of 28 (VP28), 26 (VP26), 24 (VP24), and 19 kDa (VP19), respectively. Several less prominent bands are also observed from which approximately six bands are located in the range of 30 to 65 kDa and at least seven weak protein bands ranging from 86 kDa to 130 kDa. Three major proteins bands, derived from the haemolymph are copurified with the virions and present in the range of 67 kDa to 78 kDa. Minor protein bands present in this area can not be observed in this gel (Fig. 1). The sizes found for the major WSSV proteins VP28 and VP19 are absent in the lane containing the purified nucleocapsids (Fig. 1c) and thus seem to be derived from the viral envelope or tegument. VP26 and VP24 were present in both the nucleocapsids and the virions, suggesting that they are derived from the nucleocapsid.

The content of the SDS-PAGE gel was transferred to a polyvinylidene difluoride membrane by semi dry blotting and the major viral protein bands were excised and sequenced. From VP28 and VP26 more than 40 amino acids were sequenced from the N-terminus (bold faced in Fig. 2b and 2c, respectively). The VP26 N-terminal sequence contained M E F G N L T N L D V A I I A I L S I A I I A L I V I M V I M I V F N T R V G R S V V A N. N-terminal sequencing of VP28 gave the amino acid sequence M D L S F T L S V V S A I L A I T A V I A V F I V I F R Y H N T V T K T I E t H s D, of which the threonine at position 39 and the serine at position 41 are uncertain. Both N-terminal sequences are hydrophobic (Fig. 3). The N-terminal amino acid sequence obtained via N-terminal peptide sequencing for VP24 is M H M W G V Y A A I L A G L T L I L V V I S I V V T N I E L N K K L D K K D K (SEQ ID NO 5). VP19 was found to be N-terminally blocked and a partial internal sequence of VP19 was obtained via CNBr digestion of N-terminal blocked peptide and gave the amino acid sequence I V L I S I (G/V) I L V L A V M N V (P/A/T) M G P K K D S (SEQ ID NO 6). The amino acid residue at position 7 of the VP19 partial sequence could be an G or an V, and at position 17 a P, an A or T residue.

Localization and sequence of the 24 kDa protein gene

Based on the the N-terminal protein sequence of VP24 a set of degenerate PCR primers was developed, with 5' CA*GAATTC*ATGCAYATGTGGGGNGT 3' (SEQ ID NO 13) as forward primer, and 5'

CAG*AATTC*YTTRTCYTTYTTRTCIARYTT 3' (SEQ ID NO 14) as reverse primer, both containing *Eco*RI sites (*italics*). PCR was performed using WSSV genomic DNA as template. A 133 bp-long fragment was obtained and, after purification from a 2% agarose gel, cloned into pBluescript SK+ and sequenced. The sequence of this PCR product corresponded with the N-terminal protein sequence (SEQ ID NO 5) of WSSV VP24 and was used as probe in a colony lift assay (Sambrook *et al.*, 1989) on WSSV plasmid libraries to identify the complete ORF for VP24. An 18 kbp *Bam*HI fragment hybridising with this fragment was selected for further analysis.

The complete *vp24* ORF, encompassing 627 nucleotides, and the promoter region of this gene were found on the 18 kbp *Bam*HI fragment. The translational start codon was in a favourable context (AAA*ATG*C) for efficient eukaryotic translation initiation (Kozak, 1989). In the promoter region stretches of A/T rich sequence, but no consensus TATA box, were found. A polyA signal overlapped the translation stop codon. The *vp24* ORF (SEQ ID NO 11) encoded a putative protein of 208 amino acids (SEQ ID NO 12) with an amino acid sequence containing the experimentally determined N-terminal sequence (SEQ ID NO 5) of VP24. VP24 has a theoretical size of 23 kDa and an isoelectric point of 8.7. Four potential sites for N-linked glycosylation (N-{P}-[ST]-{P}), one site for O-glycosylation (Hansen *et al.*, 1998) and 9 possible phosphorylation sites ([ST]-X-X-[DE] or [ST-X-[RK]) were found within VP24, but it is not known whether any of these modifications do occur. No other motifs present in the PROSITE database were found in VP24. Computer analysis of the 208 amino acids showed that a strong hydrophobic region was present at the N-terminus of VP24, including a putative transmembrane α-helix formed by amino acid 6 through 25. The algorithm of Garnier *et al.* (1978) predicted several other α-helices and β-sheets along the protein.

Localization and sequence of the 26 kDa protein gene

Partial WSSV genomic libraries of *Hin*dIII, and *Bam*HI were constructed in pBluescript-SK+ (van Hulten *et al.*, 2000) and terminal nucleotide sequences were obtained from many WSSV fragments. The nucleotide sequence coding for the N-terminal sequence of VP26 was present near a terminus of a 6 kb *Bam*HI fragment (Fig. 2a). The sequence surrounding the methionine start codon (AAA*ATG*G) was in conformity with the Kozak rule for efficient eukaryotic translation initiation (Kozak, 1989). Only 49 nucleotides of the untranslated leader of *vp26* could be determined, extending to the terminal *Bam*HI site (Fig. 2a).

The 6 kb *Bam*HI fragment contained an open reading frame of 555 nt including those encoding the N-terminal amino acids of VP26 (Fig. 2b). A polyA signal is present 94 nucleotides (nt) downstream of the

translational stop codon of *vp26*. This ORF (*vp26*) encoded a protein of 184 amino acids with a theoretical size of 20 kDa. The putative protein is basic with an isoelectric point of 9.4. Three potential sites for N-linked glycosylation (N-{P}-[ST]-{P}) are present and three putative O-glycosylation sites (Fig. 2b) were predicted using the program NetOglyc (Hansen *et al.*, 1998). Thirteen possible phosphorylation sites ([ST]-X-X-[DE] or [ST-X-[RK]) were found, but no other motifs present in the PROSITE database. Hydrophobicity analysis of the 184 amino acid of VP26 showed that a strong hydrophobic region is present at the N-terminus of the protein (Fig. 3a). This region contained a putative transmembrane anchor formed by amino acid 12 through 34 in the form of an α-helix. The anchor was followed by a positively charged region containing two arginines, suggesting that the orientation of the C-terminal part is to the cytoplasmic side (Sonnhammer et al., 1998). Besides the transmembrane-spanning α-helix, a potential β-sheet was found at position 127 through 141 using the algorithm of Garnier *et al.* (1978). Only one cysteine was present in the protein, indicating that no intraprotein disulfide cross-links can be formed. This cysteine was located in the C-terminal part of the protein, which was also the case in VP28.

Localization and sequence of the 28 kDa protein gene

The amino acid sequence of VP28 was not available from translations of WSSV terminal fragment sequences. Based on the N-terminal sequence of this peptide a set of degenerated primers was developed. The forward primer was 5' CA*GAATTC*TCD ATNGTYTTNGTNAC 3' (SEQ ID NO:7) and the reverse primer was 5' CA*GAATTC*ATGGAYYTN WSNTTYAC 3' (SEQ ID NO:8) with *Eco*RI sites (italics). The location of the primers on the sequence is indicated in Fig. 2c. PCR was performed using genomic WSSV DNA as template. A 128 bp-long fragment was obtained and, after purification from a 2.5% agarose gel, cloned into pBluescript SK+ and sequenced. The nucleotide sequence encoded the N-terminal protein sequence of WSSV VP28 and this 128 bp fragment was used in a colony lift assay (Sambrook *et al.*, 1989) on several WSSV plasmid libraries. A 3 kb *Hin*dIII fragment hybridized with this fragment and was further analyzed.

The complete ORF (*vp28*) of 612 nt and a promoter region of this gene was found on this 3 kb *Hin*dIII fragment (Fig. 2c). The methionine start codon (GTC*ATG*G) is in a favorable context for efficient eukaryotic translation initiation (Kozak, 1989). In the promoter region no consensus TATA box was found but stretches of A/T rich regions were present. A polyA signal was observed 55 nucleotides downstream of the translational stop codon. The ORF coded for a putative protein of 204 amino acids, which included the N-terminal sequenced amino acids. The theoretical size of this acidic protein was 22 kDa with an

isoelectric point of 4.6. Five potential sites for N-linked glycosylation (N-{P}-[ST]-{P}), two sites for O-glycosylation (Hansen *et al.*, 1998) (Fig. 2c) and 9 possible phosphorylation sites ([ST]-X-X-[DE] or [ST-X-[RK]) were found. No other motifs present in the PROSITE database are found on VP28.

Computer analysis of the 204 amino acid proteins showed that a strong hydrophobic region was present at the N-terminus of the protein (Fig. 3b), including a putative transmembrane α-helix sequence formed by amino acid 9 through 27. As in VP26, this transmembrane anchor sequence is followed by a positively charged region suggesting that the protein may have an outside to inside orientation. At the C-terminal part of the sequence another hydrophobic region was found, which might constitute a transmembrane sequence. However, the algorithm of Garnier *et al.* (1978) did not predict an α-helix at this position in VP28. The algorithm predicted a further α-helix at position 89 to 99, but no β-sheets along the protein. As in VP26 only one cysteine was present in VP28. This cysteine was also located in the C-terminal part of the protein.

Expression and analysis of recombinant *vp24, vp26* and *vp28*.

The Bac-to-Bac system (GIBCO BRL) was used for the generation of recombinant baculoviruses expressing the putative WSSV virion proteins, VP24, VP26, VP26c and VP28, in insect cells. The *vp24*, *vp26*, *vp26c* and *vp28* genes (SEQ ID NO 11, SEQ ID NO 1, SEQ ID NO 9 and SEQ ID NO 2, respectively) were cloned downstream of the polyhedrin promoter from plasmid pFastBac-D/GFP, which contains a GFP gene downstream of the p10 promoter. The recombinant viruses generated from the pFastBac-D/GFP (control), and the plasmids with *vp24*, *vp26*, *vp26c* and *vp28*, were designated *Ac*MNPV-GFP, *Ac*MNPV-WSSVvp24, *Ac*MNPV-WSSVvp26, *Ac*MNPV-WSSVvp26c, and *Ac*MNPV-WSSVvp28, respectively. All recombinant viruses expressed GFP off the p10 promoter; the latter four expressed in addition VP24 (SEQ ID NO 12), VP26 (SEQ ID NO 3), VP26c (SEQ ID NO 10) and VP28 (SEQ ID NO4), respectively, off the polyhedrin promoter.

Extracts of *Sf*21 cells infected with *Ac*MNPV-wt, *Ac*MNPV-GFP, *Ac*MNPV-WSSVvp26, and *Ac*MNPV-WSSVvp28 were analyzed in a 15% SDS-PAGE gel. In cells infected with wild type *Ac*MNPV (Fig. 4a, lane 3) a 32 kDa band was visible which represents polyhedrin. In the lanes containing extracts of *Ac*MNPV-GFP infected cells (lane 4) and cells infected with the recombinants expressing WSSV proteins (lanes 5 and 6), a GFP protein band was observed at approximately 29 kDa. The GFP expression in the cells infected with *Ac*MNPV-GFP was stronger as compared to the GFP expression in

the baculoviruses expressing WSSV proteins from the polyhedrin promoter (lanes 5 and 6). This was also readily observed after UV illumination of cells infected with the various *Ac*MNPV recombinants, where the fluorescence of GFP in *Ac*MNPV-GFP, infected cells is the strongest (not shown). The expression of the WSSV proteins from the polyhedrin promoter is significant higher compared to the expression of GFP from the p10 promoter (lane 5 and 6). A strong expression of a 21 kDa protein was observed in extracts of *Ac*MNPV-WSSVvp26 infected cells, most likely representing WSSV VP26 (lane 5). A strong expression of a 28 kDa protein was observed in the cells infected with *Ac*MNPV-WSSVvp28 (lane 6). The position of GFP in these gels was confirmed by western analysis using anti-GFP antiserum (data not shown).

Western analysis was performed on samples of wild-type and recombinant AcMNPV infected Sf21 cells electrophoresed in a SDS PAGE gel. A polyclonal antibody against WSSV virions was used to detect recombinant VP26 and VP28 (Fig. 4b). Both VP26 and VP28 were well detected in these cell extracts. VP26 was detected at 21 kDa, in conformity with the Coomassie Brilliant Blue-stained gel (fig. 4a, Lane 5; fig. 4b, Lane 5). Recombinant VP28 migrated at the same position as VP28 from WSSV virions, which is significant higher than the theoretical size of 22 kDa for this protein. The polyclonal antibody did not show major cross reactivity with insect cells (lane 2) or baculovirus (lanes 3 and 4) proteins, as observed from the very low background reaction in these samples.

Extracts of *Sf*21 cells infected with AcMNPV-WSSVvp26c and AcMNPV-WSSVvp24 were analyzed in a 15% SDS-PAGE gel. A low molecular weight marker and purified WSSV virions were also analyzed in the same gel. A weak band at 29 kDa was observed in the lanes containing the AcMNPV-WSSVvp26c and AcMNPV-WSSVvp24 infected cells, representing GFP, which was clearly observed after UV illumination of the infected cells. Furthermore in the lane with the AcMNPV-WSSVvp26c infected cells, a strong band was observed at 26 kDa at the same position in the gel as the 26 kDa band in the WSSV virions. In the lane containing the cells infected with AcMNPV-WSSVvp24, a clear band was observed at 24 kDa, corresponding with the position of the 24 kDa protein in the WSSV virions. To confirm that the 26 kDa band in AcMNPV-WSSVvp26c infected cells and the 24 kDa band in AcMNPV-WSSVvp24 infected cells correspond to the 26 kDa and 24 kDa protein in the WSSV virions, a Western blot was made of this gel using a polyclonal antibody against WSSV virions. The 26 kDa band in AcMNPV-WSSVvp26c infected cells and the 24 kDa band in AcMNPV-WSSVvp24 infected cells were well detected.

Relatedness of VP26 and VP28

Homology searches with WSSV VP24, VP26, VP26c and VP28 were performed against GenBank/EMBL, SWISSPORT and PIR databases using FASTA, TFASTA and BLAST. No significant homology could be found with the sequences in the GenBank, neither with baculovirus envelope or capsid proteins, nor with structural proteins from other large DNA viruses.

Neutralisation experiment

The titer of the virus stock was obtained in a titration experiment. The virus stock was diluted 1 x 10^7 till 5 x 10^{11} times and for each dilution 10 µl was injected intramuscular in 10 shrimps (*Penaeus monodon*, 3 – 4 month old). The 1 x 10^8 dilution of the stock WSSV solution resulted in a mortality of 50 % after 7 – 12 days and was used in further experiments.

4 groups of shrimp were used in the neutralisation experiment:

Group #	Group name	Injection	# shrimp
1	Negative control	330 mM NaCl	10
2	Positive control	WSSV	10
3	Pre-immune serum	WSSV + pre-immune serum	15
4	VP28 antiserum	WSSV + VP28 antiserum	15

The total amount of virus administered per shrimp is constant in all groups and equals 10 µl of the 1 x 10^8 dilution of the virus stock. The concentration of serum in group 3 and 4 is the same (per injection: 1µl WSSV and 9 µl of serum). After injection the shrimp were monitored for 4 weeks and dead shrimp were examined for the presence of WSSV by electron microscopy. The results are shown in Figure 5.

None of the shrimp in group 1, the negative control, died of WSSV, therefore the mortality is 0%. In the positive control (group 2), 100% mortality was reached after 23 days. The group where pre-immune serum (that is serum taken before the rabbit was injected with VP28 proteins) was added to WSSV (group 3) reached 100% mortality in 25 days. When VP28 antiserum was added to WSSV (group 4), all shrimp survived resulting in 0% mortality. These results show that VP28 antiserum can neutralize WSSV infection in *P. monodon*.

Protein vaccination

Groups 3-6 were injected with 5 μl (vaccination) and 10 μl (booster) of the different protein solutions:. For the vaccination group 3 received 2.5 μg VP28 protein, group 4 received 3.6 μg VP26c protein and group 5 received 0.7 μg of VP24 protein. Group 6 received a mix consisting of equal volumes of VP28-, VP26c- and VP24 solution, resulting in a total amount of 2.7 μg protein. For the booster the shrimps recieved higher amounts of protein: 9.6 μg of VP28 protein for group 3, 5.7 μg of VP26c protein for group 4, 5.9 μg of VP24 protein for group 5 and a total amount of 7.1 μg protein for group 6. All groups of shrimp were injected with 10 μl of a 1 x 10^8 dilution of the stock WSSV solution.

The results of the vaccination are presented in Figure 6. None of the shrimp in group 1, the negative control, died of WSSV, therefore the mortality is 0%. In group 2, shrimp start dying of WSSV infection after 1 day and mortality is increasing. Although these shrimps received the same dosage of WSSV virus as the shrimp in the neutralisation experiment, the shrimp in group 2 are dying earlier. This is probably the result of stress caused by the multiple injections that the shrimp received in this experiment. In group 3-5 (shrimps vaccinated with VP24, VP26c and VP28, respectively) mortality was delayed, while in group 6 (shrimps vaccinated with a mixture of VP24 VP26c and VP28), none of the shrimps died of WSSV, hence the mortality is 0%. Optimising the dosage of the individual proteins in vaccination will also result in an increased protective effect against WSSV infection.

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Claims:

1. Vaccine for use in prophylaxis and/or treatment of White Spot Syndrome in crustaceans comprising a pharmaceutically acceptable carrier and one or more proteins, said proteins comprising at least one of the amino acid sequence depicted in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:10 or SEQ ID NO:12, respectively .

2. Vaccine according to claim 1 characterised in that vaccine comprises a mixture of a protein having the amino acid sequence depicted in SEQ ID NO 3 or 10, a protein having the amino acid sequence depicted in SEQ ID NO 4 and a protein having the amino acid sequence depicted in SEQ ID NO 12.

3. Vector vaccine for use in prophylaxis or treatment of White Spot Syndrome in crustaceans characterised in that said vaccine comprises an attenuated bacterium or virus, said bacterium or virus comprising in its genome a heterologous nucleic acid sequence encoding a WSSV protein, said WSSV protein comprising at least one of the amino acid sequence as depicted in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:10 or SEQ ID NO:12, respectively.

4. A structural protein derived from White Spot Syndrome Virus characterised in that the amino acid sequence of said protein comprises at least one of the amino acid sequences depicted in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:10 or SEQ ID NO:12, respectively

5. Nucleic acid sequence encoding a structural protein according to claim 4.

6. Nucleic acid sequence according to claim 5 comprising the nucleic acid sequence depicted in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:9 or SEQ ID NO:11.

7. Use of a structural protein according to claim 4 as a medicament.

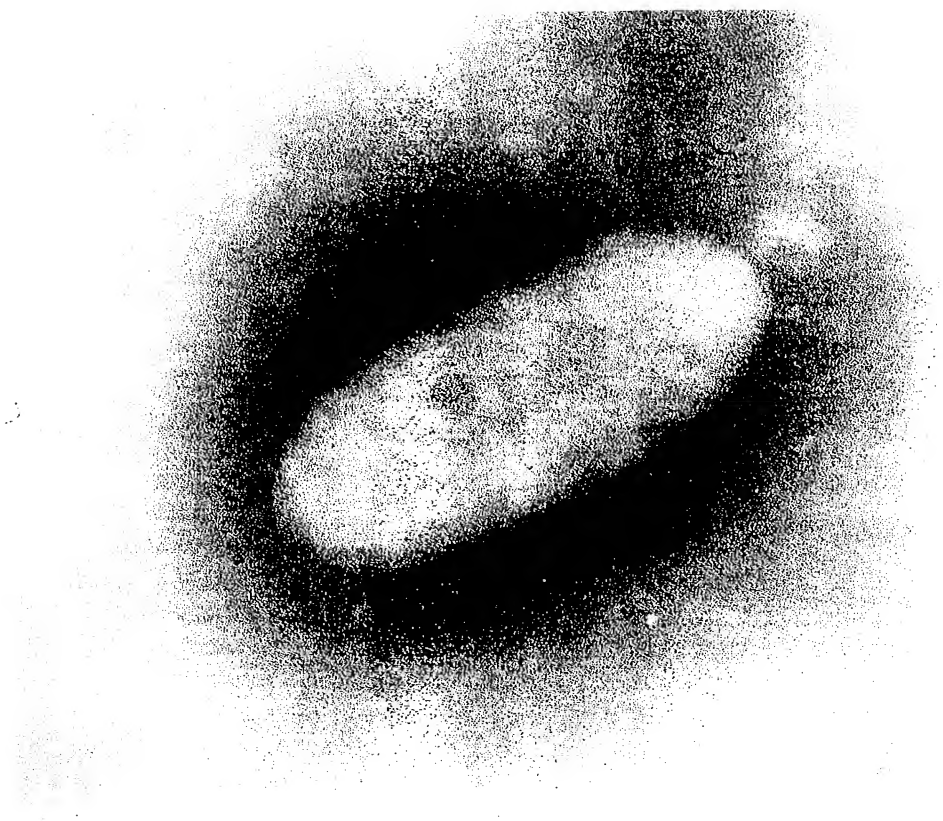
8. Pharmaceutical composition comprising a pharmaceutically acceptable carrier and at least a structural protein according to claim 4 or at least a nucleic acid sequence according to claims 5 or 6.

9. Antibodies raised against a structural protein according to claim 4.

10. Vaccine or pharmaceutical formulation comprising a pharmaceutically acceptable carrier or vehicle and at least an antibody according to claim 9.

11. Diagnostic kit for detection of WSSV characterised in that said kit comprises a nucleic acid sequence according to one of the claims 5 or 6 or an antibody according to claim 9.

Figure 1A



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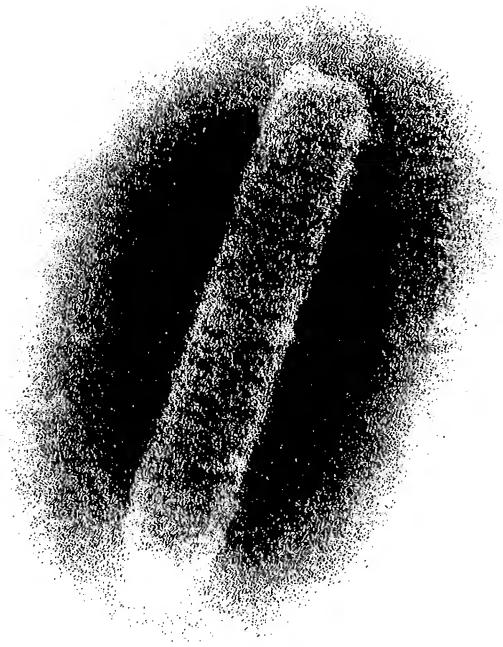

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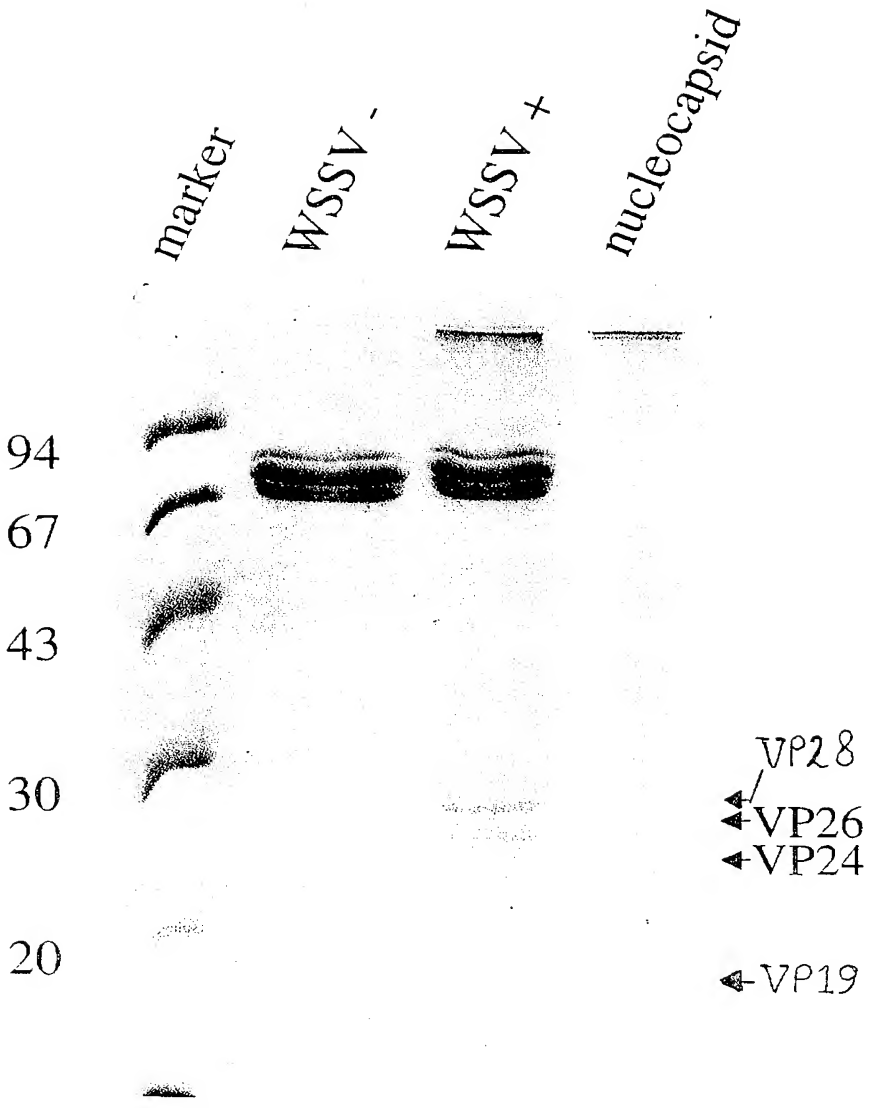
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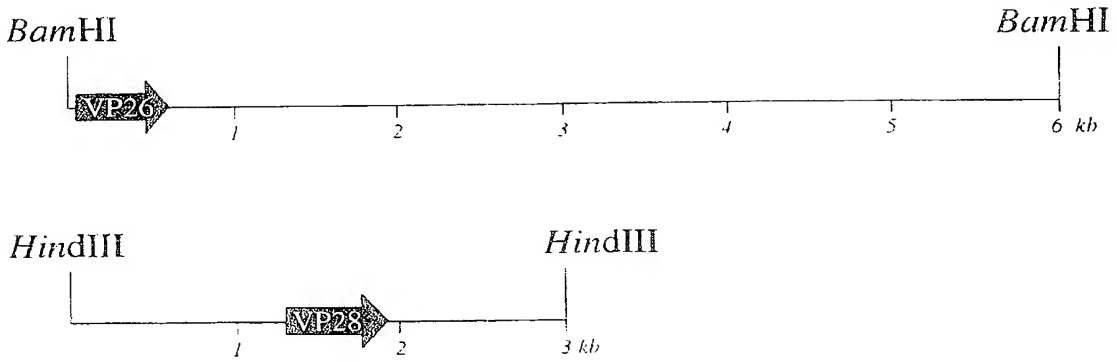

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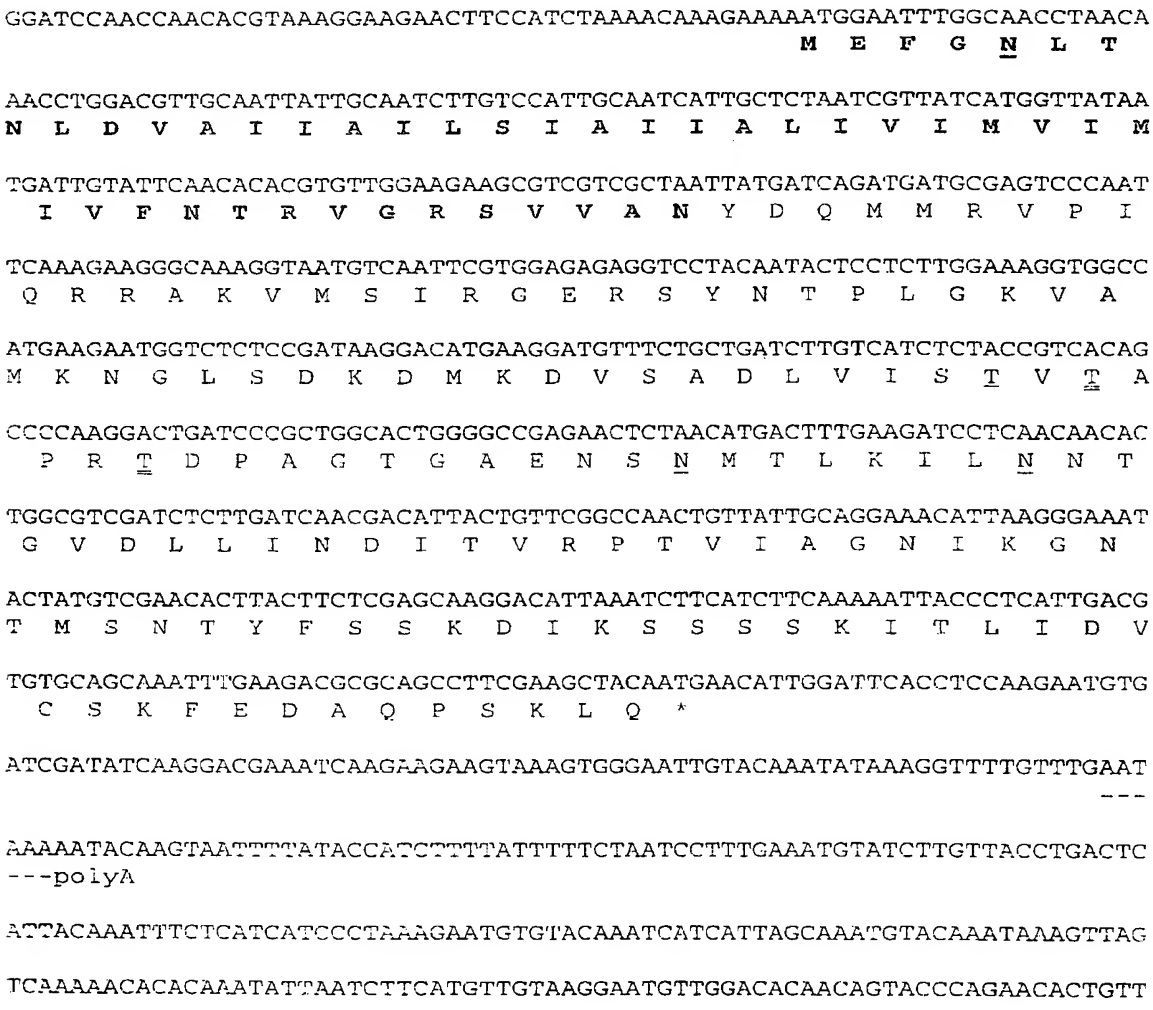
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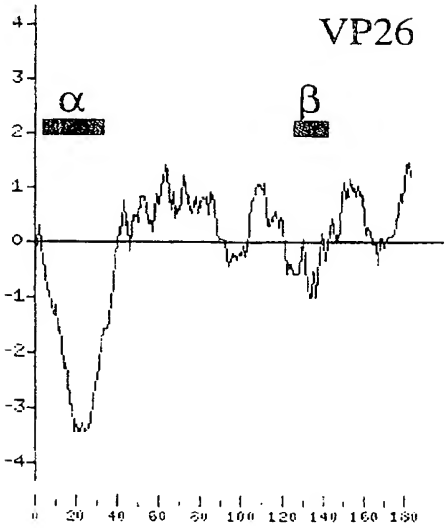

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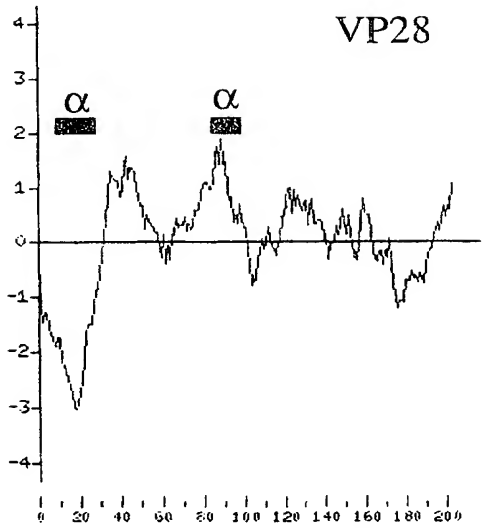

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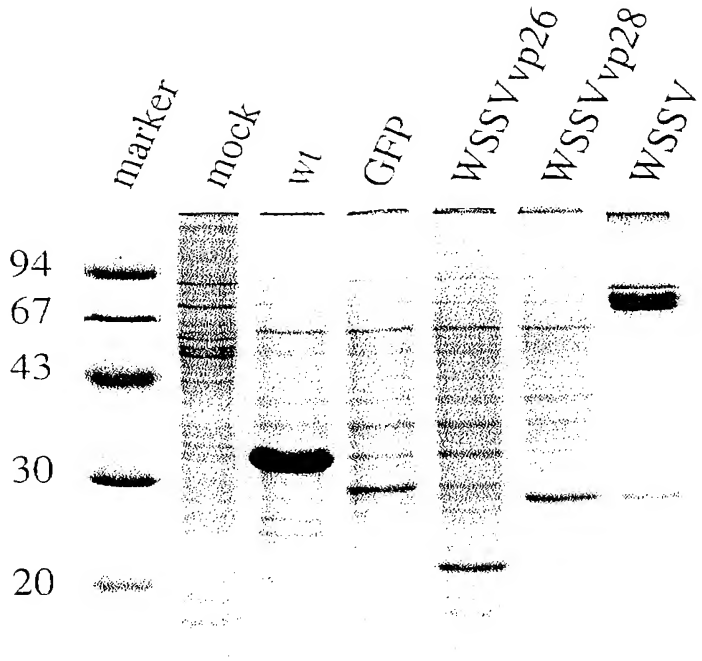

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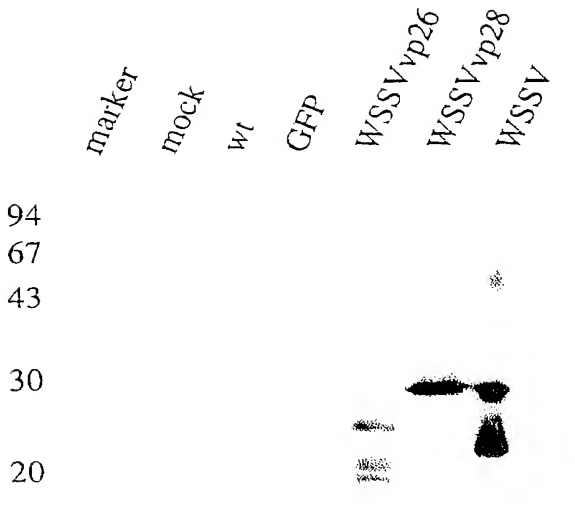

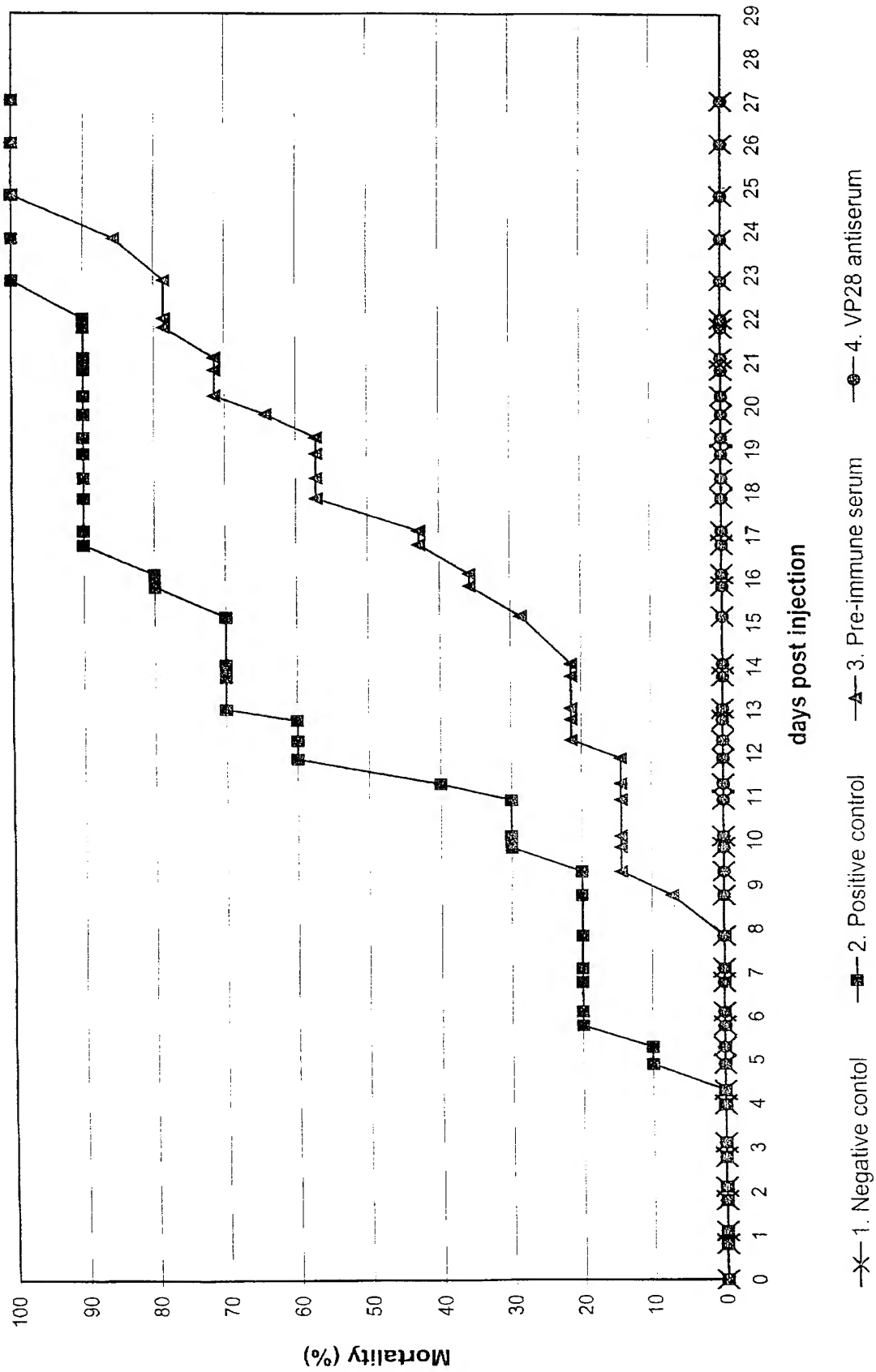

Fig.5: Neutralization experiment

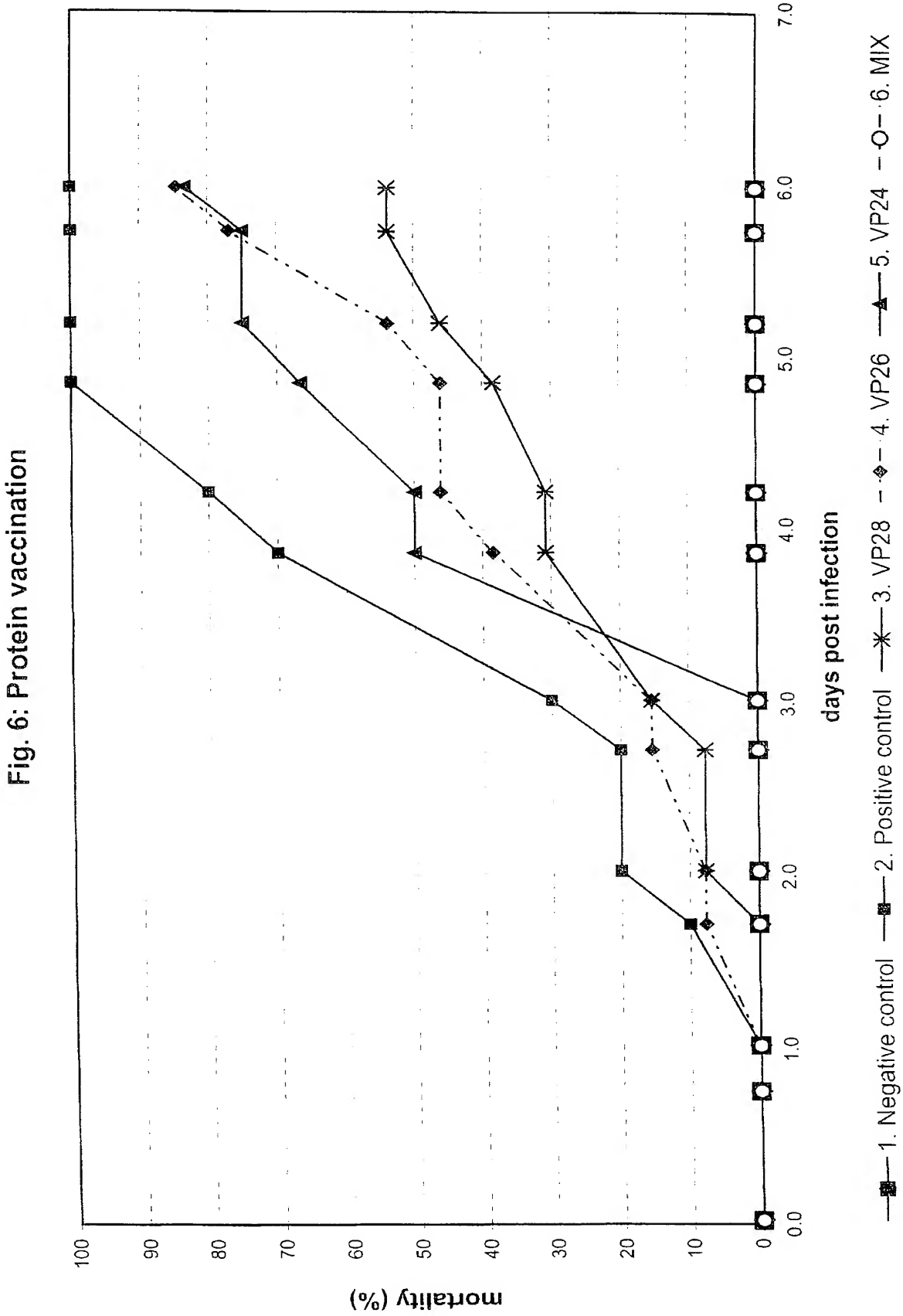

Fig. 6: Protein vaccination

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Asn Ser Asn Met Thr Leu Lys Ile Leu Asn Asn Thr Gly Val Asp Leu
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Phe Lys Met Thr Asp Val Ser Phe Asp Ser Asp Thr Leu Gly Lys Ile
 65                  70                  75                  80

Lys Ile Arg Asn Gly Lys Ser Asp Ala Gln Met Lys Glu Glu Asp Ala
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Asp Leu Val Ile Thr Pro Val Glu Gly Arg Ala Leu Glu Val Thr Val
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Gly Gln Asn Leu Thr Phe Glu Gly Thr Phe Lys Val Trp Asn Asn Thr
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Val Phe Asn Thr Arg Val Gly Arg Ser Val Val Ala Asn Tyr Asp Gln
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Gly Glu Arg Ser Tyr Asn Thr Pro Leu Gly Lys Val Ala Met Lys Asn
 65                  70                  75                  80

Gly Leu Ser Asp Lys Asp Met Lys Asp Val Ser Ala Asp Leu Val Ile
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Ser Thr Val Thr Ala Pro Arg Thr Asp Pro Ala Gly Thr Gly Ala Glu
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Asn Ser Asn Met Thr Leu Lys Ile Leu Asn Asn Thr Gly Val Asp Leu
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Lys Gly Asn Thr Met Ser Asn Thr Tyr Phe Ser Ser Lys Asp Ile Lys
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Gly Gln Gly Thr Ser Asp Ser Glu Leu Val Lys Lys Lys Gly Asp Ile
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Lys Pro Thr Val Leu Lys Lys Asn Arg Ile Asn Thr Leu Val Phe Gly
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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/EP 00/07290

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IPC 7 C12N15/34 C07K14/01 A61K39/12 C07K16/08 C12Q1/70
G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 785 255 A (WANG CHUNG HSIUNG :LU CHU FANG (TW); KOU GUANG HSIUNG (TW)) 23 July 1997 (1997-07-23) claims 3,12,20 ---	5
X	LO C-F ET AL: "Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus" DISEASES OF AQUATIC ORGANISMS, vol. 35, no. 3, 26 February 1999 (1999-02-26), pages 175-185, XP000878518 ISSN:0177-5103 cited in the application figures 2,3 --- -/--	5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 December 2000	02/01/2001

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/EP 00/07290

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YANG F ET AL: "A simple and efficient method for purification of prawn baculovirus DNA" JOURNAL OF VIROLOGICAL METHODS, vol. 67, no. 1, 1997, pages 1-4, XP000878721 ISSN:0166-0934 cited in the application figure 2 ---	5
P,X	VAN HULTEN MC ET AL.: "Identification of two major virion protein genes of white spot syndrome virus of shrimp" VIROLOGY, vol. 266, no. 2, 20 January 2000 (2000-01-20), pages 227-236, XP002155538 ORLANDO US figure 2 -----	3-6

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ial Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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